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Original Research

Isolation of Mare's Milk Oligosaccharide Fraction of Colostrum, Transitional, and Mature Phases Promotes In Vitro Oxidative Burst in Murine Macrophages

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ABSTRACT

Present preliminary findings demonstrate in vitro stimulation of splenic lymphocytes of BALB/c mice after exposure to oligosaccharide fractions isolated from mare's milk during colostrum, transitional, and mature lactation periods, which shows immunostimulatory action of oligosaccharide content present in mares' milk. Oligosaccharide fractions caused more pronounced oxidative burst at a much lower concentration (0.1 μg/mL) compared with that shown by levamisole (standard immunostimulant) at a much higher concentration of 10 μg/mL. In this study, it was observed that oligosaccharides fraction of colostrum phase attaining high potency of immunostimulatory property.

1. Introduction

Milk is the nature's designer food for the newborn, and it contains many nutritious and bioactive components, which are responsible for the homeostasis and health [1,2]. Mammalians having four phases of lactation period, which differs in the composition and volume of milk viz: colostrum, transitional, mature and involutional lactation phase [3–5]. Colostrum is secreted for the first 3–5 days after delivery, transitional milk until the end of the second week, mature milk during full lactation, and involutional milk at the end of lactation [6,7]. Mammalian milk or colostrum in addition to lactose usually contains a variety of many glycoproteins, glycopeptides, and number of free oligosaccharides [8]. Numerous oligosaccharides have been detected in the milk or colostrums of many mammalians including equines, bovine, and marine mammals [9–12]. Milk oligosaccharides serve as prebiotics to stimulate growth of beneficial intestinal bacteria such as bifidobacteria in neonates. Modulation of the postnatal immune system is a beneficial consequence because of the development of a balanced intestinal microbiota. Additionally, milk oligosaccharides have been reported to bind to certain pathogenic microorganisms thereby limiting their virulence. This behavior lowers the risk of diseases such as diarrhea, meningitis, and otitis media in infants [13]. Milk oligosaccharides have shown diverse biological activities such as anticancer immunostimulant, antitumor, anticancer, anti-inflammatory, hypoglycemic, anticomplementary, antiviral, and antimicrobial [14,15]. It is observed that those oligosaccharide moieties containing mannose or galactose sugars units, then it facilitates the wound healing activity by activating macrophages which orchestrate the

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release of various bioactive substances that modulate the immune response and tissue inflammation [16,17]. Biological activity of mare's milk is well described in ancient medical literature of India and in some recent literature. The distribution of different types of milk oligosaccharides (fucosyl oligosaccharides, sialated oligosaccharides, fucosyl–sialated oligosaccharides, and other normal lactose-containing oligosaccharides) varies dynamically during different phases of lactation [18,19]. These milk oligosaccharides are responsible for immunologic effect and play potent role in inhibition of microorganism [20]. Some compounds of natural (including oligosaccharides) or synthetic origin termed as immune potentiating agents or immunomodulators may nonspecifically induce reactive oxygen species (ROS) production by activated immune cells which act against the stress conditions (caused by environment or parasite), involved in cell signaling (at low levels), maintaining homeostasis (at higher levels), cytotoxic mechanisms (which helps in killing the phagocytosed pathogens), and pathology [21]. In 1999, Saksena et al [22] have reported the immunostimulant activity of buffalo milk oligosaccharides using mouse model as revealed by increase in the hemagglutination titer, delayed-type hypersensitivity reaction, and plaque-forming cell counts in mice. Spearman [23] worked on the mannan-oligosaccharide supplementation effect on the immune status of mares and their foals, and he found that first 56 days of lactation significant incensement of immunoglobulin (Ig) G (IgG) and IgA contents and tended to increase IgM content in the colostrums of mare. Then, authors thought, if mannan-oligosaccharide supplementation can increase the immune status of mares and their foals, then those oligosaccharides which are natural content of milk may also be immunostimulating in nature. Human milk contains a high amount of unbound complex oligosaccharides (5–10 g/L) that carry one or more Lewis antigen glycans, and we hypothesized that they compete with gp120 for Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) binding. Here, we show that in two independent assays, physiological concentrations of human milk oligosaccharides significantly reduce gp120 binding to DC-SIGN by more than 80%. These results may provide an additional explanation for the inhibitory effects of human milk on human immunodeficiency virus 1 (HIV-1) mother-to-child transmission. Identifying the specific milk oligosaccharides that interact with DC-SIGN may guide the development of glycan-based drugs that prevent transmission of HIV-1 and other pathogens that use DC-SIGN as an entry point. However, blocking DC-SIGN may be a two-edged sword [24].

Keeping these significant properties in mind, that is, ancient Indian literature about mare's milk, immunostimulant activity of buffalo's milk oligosaccharide, and supplementation effect of oligosaccharide on the immune status of mares, an attempt was made to isolate oligosaccharides from mare's milk at different lactation periods, that is, colostrum, transitional, and mature lactation phases and study their effect on macrophage functions of BALB/c mice to further investigate their potential as immunostimulants or immune adjuvants.

2. Material and Methods

2.1. Instruments Used for Isolation of Oligosaccharide Fraction

All chemicals, solvents, and reagents were of analytical grade, Buchi Rotavapor R-200/R205 was procured from Buchi Labortechnik, Switzerland, CT 60e (HETO) lyophilizer purchased from Heto Equipments (Denmark), and cooling with centrifuging machine C-23 JJRCI 763 was purchased from Remi Instruments, Goregaon-East Mumbai (India). Sephadex G-25 gel permeation chromatography unit was of GE Healthcare Bio-Sciences Ltd, New Delhi (India). Antioxidant activities were performed on Photochem analyzer which was procured from Analytik Jena AG (Germany). High-performance liquid chromatography (HPLC) grade water was of Spectrochem Pvt Ltd, Mumbai (India); Methanol was obtained from Merck Specialties Pvt Ltd, Mumbai (India). Phenol and sulfuric acid were purchased from Laba Chemie Pvt Ltd, Mumbai (India).

2.2. Isolation of Oligosaccharide Fractions

For in vitro immunologic assessment of mare's [25] milk oligosaccharide fractions, samples were collected from three different phases after parturition, that is, colostrum phase (first to fifth day), transitional phase (sixth to 14th day), and mature phase (15th–31st day) at animal house, Directorate of Animal Husbandry, Lucknow (India). Of five mares used, three mares were attaining their first lactation period and another two in their second lactation period. All five mares were included at each stage, and the milk from each of these five mares was pooled and collected up to 2.0 L (400 ± 100 mL/d up to 2.0 L). The mare was on normal diet (green grass with leaves and water) and atmospheric condition, milking process was hand made from both teats once a day, collection of milk was up to 100 ± 20 mL from each mare per day, and milk was kept at -20°C until use. For assessment of biological activity of milk, milk was centrifuged at -4°C for 20 minutes at 6,000 $\times$ *g*, and solidified lipid layer was removed by filtration through glass wool column in cold atmospheric condition. Ethanol was added to make up final concentration of 70% and left overnight at 0°C. The white precipitate formed mainly of lactose and protein was removed by centrifugation at 6,000 $\times$ *g* and washed twice with 68% ethanol at 0°C. The supernatant was passed through a microfilter (0.24 μ) to remove remaining lactose and lyophilized to get crude oligosaccharide mixture (2.85 g from colostrum, 2.43 g from transitional, and 2.05 g from mature periods), which was further purified on Sephadex G-25 chromatography [22].

2.3. Size Exclusion Chromatography of Crude Mare's Milk Oligosaccharide

For purification of oligosaccharide fraction, 2.0 g of milk oligosaccharide fraction was packed in a column (1.6×40 cm) (void volume = 25 mL) and eluted with triple distilled water as mobile phase at 10 mL per 30-minute flow rate. Eluted fractions under peaks II, III, IV, and V gave positive phenol–sulfuric acid test (mature period), which were further purified on Sephadex G-25 chromatography

[22], showed the presence of oligosaccharide mixture. The fractions were pooled and lyophilized for chemical analysis and evaluation of biological activity. After pooling of each pure oligosaccharide fraction of every lactation period, 1.35 g of colostrum, 1.02 g of transitional, and 0.983 g of mature oligosaccharide mixtures were obtained. The results of Sephadex G-25 chromatography are given in chromatogram (Fig. 1) and total oligosaccharides obtained are given in Table 1.

2.4. HPLC–Evaporated Light-Scattering Detection of Milk Oligosaccharides

Crude milk oligosaccharide obtained from Sephadex G-25 chromatography was further analyzed through reverse phase–high performance liquid chromatography–evaporated light-scattering detection (RP-HPLC-ELSD) method. For this purpose, accurately weighed 200 mg powder of crude extract was mixed with 20 mL of 50% acetonitrile in water to prepare 10 mg/mL solution. The prepared extract was filtered through a 0.22 μm Fluoropore membrane (Millipore, New Bedford, MA) before injection into the HPLC system. The samples were prepared and analyzed in triplicate, and the contents of the analytes were determined from the corresponding calibration curves. Chromatographic separation was carried out on a Simadzu instrument equipped with a PL-ELS 2100 controller (Polymer Laboratories), ELS detector, and Supelco C18 column (250 × 4.6 mm, 5 μm particle size) were used for the separation of oligosaccharides. High-performance liquid chromatography conditions were as follows: eluant A, 0.5 acetic acid in water; eluant B, acetonitrile; gradient, 0–6 minutes (10%–15% B), 6–25 minutes (15%–25% B), 25–35 minutes (25%–30% B) and then equilibrated with 10% B for 5 minutes at a flow of 1 mL/min. Evaporated light-scattering detection was set to a probe temperature of 85 C, a gain of 7, and the nebulizer gas nitrogen adjusted to 2.5 bar (Fig. 2). Data acquisitions were performed using LC solution software version (Simadzu).

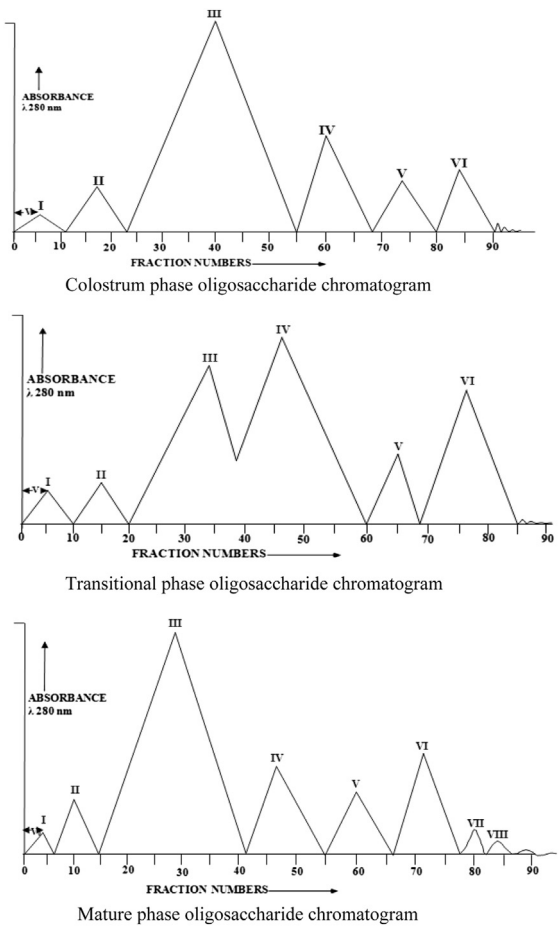

Fig. 1. Colostrum, transitional, and mature phases' oligosaccharides chromatogram of Sephadex G-25.

2.5. Evaluation of Immunomodulatory Activity of Mare's Milk Oligosaccharide

Medium RPMI-1640 (Sigma) devoid of phenol red and phosphate-buffered saline (PBS) fortified with 1% antibiotic–antimycotic cocktail (Sigma) and 10% fetal bovine serum (GIBCO) was used for in vitro cell culture. 2′,7′-dichlorofluorescin diacetate (DCF-DA) (Sigma) was used to estimate the production of ROS.

2.6. Animals Used and Sample Preparation

Inbred BALB/c mice (18–20 g) were kept in a temperature- and humidity-controlled room (25–26°C; relative humidity, 60%–80%) under proper hygienic conditions at 12-hour light and dark cycle in the Animal House of Central Drug Research Institute (CDRI), Lucknow, India and given free access to the normal diet and water ad libitum. The animal handling and experimental protocols employed in the present study were duly approved by the CDRI Animal Ethics Committee. The milk oligosaccharide sample from each lactation phase was dissolved in dimethyl sulfoxide for in vitro study.

2.7. Estimation of Intracellular ROS Production From In Vitro Activated Peritoneal Macrophages

Reactive oxygen species content in the peritoneal macrophages of mice was determined by a fluorometric assay using dichlorofluorescin (DCF) in fluorescence-activated cell sorting (FACS) flow cytometry [26]. The macrophages were collected aseptically from the peritoneal cavities of BALB/c mice and plated into flat bottom 96-well culture plate (Nunc). Cells (1×10^6 cells/well) were allowed to adhere to plastic bottom for 2 hours at 37°C in 5% CO_2 in air [27], washed, and the fresh medium was added. The cells were then exposed to each oligosaccharide fraction isolated from mare's milk at colostrum, transitional, and mature lactation phases at four different concentrations (100, 10, 1.0, and 0.1 μg/mL each). For comparison, levamisole, a known immunostimulatory drug [28–30], was also used in triplicate wells at an optimal concentration of 10 μg/mL. After an overnight incubation at 37°C in CO_2 incubator, cells were harvested into separate tubes and washed thrice in PBS. For probe loading, cells were incubated with the DCF-DA (1 μM)

Table 1
Sephadex G-25 (1.6 × 40 cm) chromatography of mare's milk oligosaccharide fraction at different lactation phases.

Period of Lactation and Milk Collected (2 L)	Crude Mare's Milk Oligosaccharide Taken (g)	Mobile Phase Used	Fraction Numbers	Phenol–H_2SO_4 Test for Sugar	Compound Obtained (g)	Pure Mare's Milk Oligosaccharide Obtained (g)
Colostrum milk (1st–5th day)	2.0	Glass triple distilled water at 10 mL/min flow rate	1–11	−ve (I)	0.10	1.35
			12–22	+ve (II)	0.13	
			23–55	+++ve (III)	0.85	
			56–68	++ve (IV)	0.21	
			68–80	+ve (V)	0.16	
			81–91	−ve (VI)	0.21	
Transitional milk (6th–14th day)	2.0	Glass triple distilled water at 10 mL/min flow rate	1–9	−ve (I)	0.11	1.02
			10–19	+ve (II)	0.12	
			20–38	++ve (III)	0.32	
			39–59	+++ve (IV)	0.43	
			60–68	+ve (V)	0.15	
			69–85	−ve (VI)	0.35	
Mature milk (15th–31st day)	2.0	Glass triple distilled water at 10 mL/min flow rate	1–6	−ve (I)	0.08	0.98
			7–14	+ve (II)	0.11	
			15–41	++ve (IV)	0.55	
			42–54	+++ve (V)	0.19	
			55–66	+ve (V)	0.13	
			67–77	−ve (VI)	0.21	
			78–82	−ve (VII)	0.04	
			83–87	−ve (VII)	0.03	

for 15 minutes at 37°C, washed twice in PBS, and transferred to FACS tubes. Reactive oxygen species level in individual living cell was determined by measuring their fluorescence intensity on FACSCalibur (Becton Dickinson). Data were analyzed by CellQuest Software (Becton Dickinson), and mean ROS values were determined for cell population.

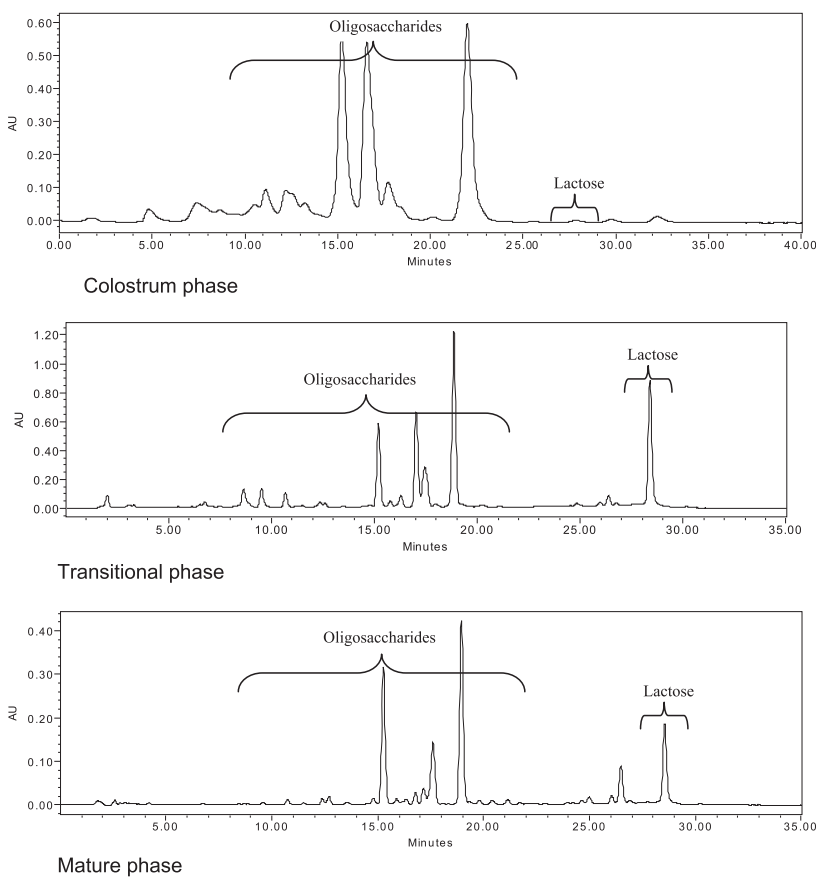

Fig. 2. High-performance liquid chromatography–evaporated light-scattering detection chromatogram of colostrum, transitional, and mature phases' oligosaccharides fraction.

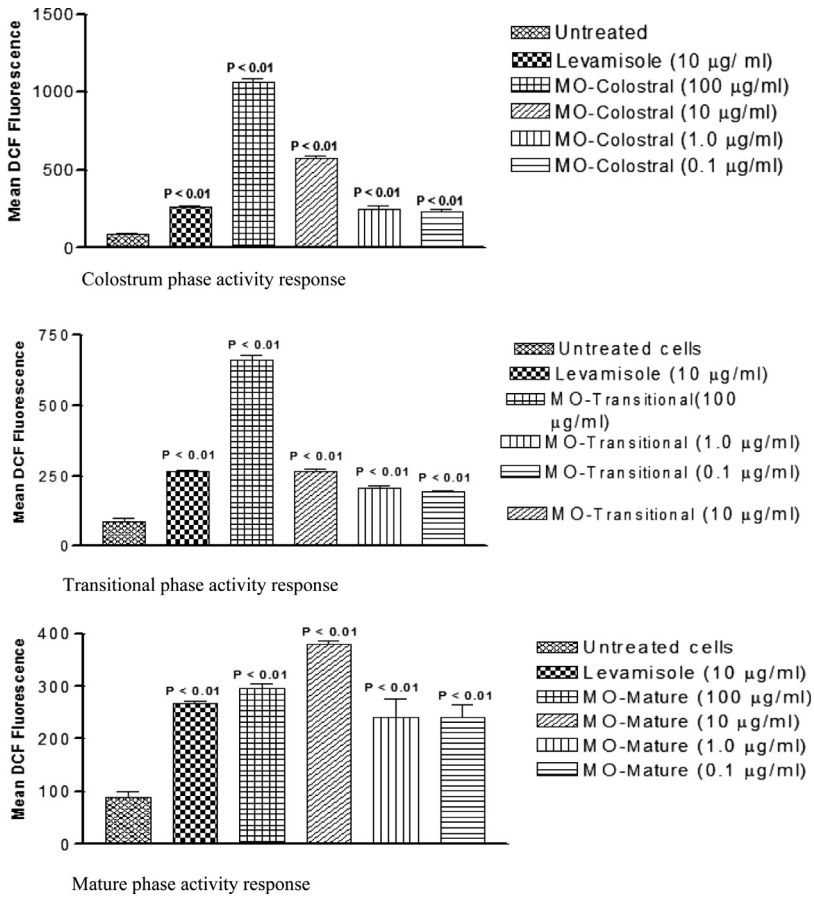

Fig. 3. Immunomodulatory response of colostrum, transitional, and mature phases MO with levamisole (as a standard drug). DCF, dichlorofluorescin; MO, milk oligosaccharides.

2.8. Statistical Analysis

The results have been presented as the mean ± standard error, and statistical significance was determined using GraphPad Prism software (version 3.0) through one-way analysis of variance followed by Dunnett multiple comparison test. Values of $P < .05$ were considered as indicative of significance; $P < .01$ was considered as highly significant, whereas $P > .05$ was considered as not significant.

3. Results

The ROS levels were found to be significantly ($P < .01$) elevated in the macrophages stimulated with oligosaccharides as compared with that of untreated cells or the levamisole used in the present study. Although levamisole induced ROS generation significantly higher ($P < .01$) than untreated cells, however, the effect was remarkably enhanced in presence of all the three oligosaccharides of the four different concentrations (100, 10, 1.0 and 0.1 μg/mL) of oligosaccharide, maximum ROS stimulation occurred at the highest tested concentration of 100 μg/mL in case of colostrum and transitional phases while mature phase oligosaccharide showed maximum macrophage stimulation at 10 μg/mL concentration (Fig. 3). The Fig. 3 show a significant dose dependent increase ($P < .01$) in the ROS level after in vitro exposure of mouse peritoneal macrophages to various log concentrations of oligosaccharides, while mature phase figure not showing a dose dependent response. The activities of mare's oligosaccharide fraction of colostrum phase at lower concentrations of 1.0 and 0.1 μg/mL were more or less comparable with that of 10 μg/mL of levamisole; however, oligosaccharide from transitional and mature phases at 10 and 100 μg/mL, respectively, had comparable activities with 10 μg/mL of levamisole. However, the increase in ROS generation by oligosaccharide content of colostrum and transitional phases at 100 and 10 μg/mL were much higher than levamisole.

4. Discussion

Nature has given us a lot of flora and fauna as reservoir of life-saving compounds to combat unfavorable stress conditions. These compounds of natural (including oligosaccharides) and some of synthetic origin termed as immune potentiating agents or immunomodulators have the capacity to either stimulate or suppress the immune cells of our body and thereby regulate the internal environment. The main target of the immunomodulators primarily is macrophage [31], which plays a central role in the induction

and regulation of immune response. Activated macrophages cause increased phagocytosis, intracellular killing of pathogens by producing effector molecules such as ROS, nitric oxide, and cytokines [21,32–34]. Reactive oxygen species are oxygen-centered free radicals, hydroxyl radicals, or reactive nonradical compounds [35] and are well documented to be associated with the innate immune response toward infection where they are produced at high levels within phagocytes to kill internalized organisms. Reactive oxygen species acts against the stress conditions (caused by environment or parasite), involved in cell signaling (at low levels), maintaining homeostasis (at higher levels), cytotoxic mechanisms (which helps in killing the phagocytosed pathogens), and pathology [21]. Superoxide ($O_2^{\bullet -}$) appears to play a central role in living cells. It is formed from the one electron reduction of molecular oxygen (O_2) mediated by electron leakage from the mitochondrial respiratory chain or by enzymes such as NADPH oxidase (nicotinamide adenine dinucleotide phosphate (NADP) carrying electrons and bonded with a hydrogen (H) ion; the reduced form of NADP) and xanthine oxidase [36,37]. During this reaction, there is a high consumption of oxygen, and thus, the process is termed as the "respiratory burst" [38]. NADPH oxidase is the major enzyme responsible for $O_2^{\bullet -}$ production within phagocytic cells such as neutrophils and macrophages. These cells use $O_2^{\bullet -}$ to kill ingested organisms.

The preliminary results obtained in the present study with mare's milk demonstrated that oligosaccharides promote cellular immune response as observed in vitro both in terms of cellular proliferation and reactive oxidative burst. The oligosaccharide fraction at various concentrations from 100 to 0.1 μg/mL significantly ($P < .01$) stimulated the peritoneal macrophages to synthesize increased level of ROS which may be interpreted as an activation of one of the innate immune defense mechanism. The fraction of colostrum and transitional lactation phases at 100 mg/mL concentration and that of mature phase at 10 mg/mL markedly activated the macrophages to synthesize ROS, which was significantly higher than the standard drug levamisole (a synthetic phenylimidazolthiazole) a potent anthelminthic [39] and immune-enhancing agent [32] when it was used at 10 mg/mL concentration. The higher response of milk isolates compared with the levamisole could be due to higher immune-stimulating effects of oligosaccharides. Second, each fraction contained a number of oligosaccharides and the net action is due to all these, whereas, on the contrary, levamisole is a pure compound. All the three oligosaccharides had activity even at a lower concentration of 1.0 and 0.1 μg/mL. In general, it was observed that murine macrophages were maximally activated by the oligosaccharide fraction collected at the colostrum phase, which probably denotes the presence of these compounds at the highest concentration in the colostrum milk. The ability of macrophages to kill pathogenic microbes is probably one of the most important mechanisms of protection against diseases. Increased respiratory burst can be correlated with increased killing activity [40]. Interestingly, levamisole was comparatively a poor ROS inducer compared with oligosaccharides. Thus, the oligosaccharides from mare's milk show great promise as an immunostimulant and need further exploitation for their effect on various host immune parameters to know its worthiness as immunoprophylactic agent against various parasitic and other pathogenic diseases and as adjunct to chemotherapeutic treatment of these diseases.

5. Conclusion

In view of the previously mentioned results, the milk fractions need to be further explored in detail for its biological activity in vivo on various immune parameters to evaluate its usefulness as a drug for boosting the host's immune response against various diseases and as adjunct to drug or vaccine.

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